Evaluation of Peripheral Immune Activation in Amyotrophic Lateral Sclerosis

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Research

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Abstract

Background: Accumulating evidence has revealed that immunity plays an important role in amyotrophic lateral sclerosis (ALS) progression. However, the results regarding the serum levels of immunoglobulin and complement are inconsistent in patients with ALS. Although immune dysfunctions have also been reported in patients with other neurodegenerative diseases, few studies have explored whether immune dysfunction in ALS is similar to that in other neurodegenerative diseases.

Methods: Serum levels of immunoglobulin and complement were measured in 245 patients with ALS, 65 patients with multiple system atrophy (MSA), 60 patients with Parkinson's disease (PD), and 82 healthy controls (HCs). A meta-analysis including data from this study was performed to evaluate the differences in the levels of immunoglobulin and complement between ALS patients and HCs. The serum levels of immunoglobulin and complement were compared between patient groups and HCs or between ALS patient groups established by age at onset, site at onset, disease duration, or disease severity. The correlations between the levels of immunoglobulin and complement and the clinical characteristics of ALS were analysed using Spearman correlation analysis.

Results: The pooled results showed that patients with ALS had higher C4 levels than did HCs, and no significant differences between these two groups in IgG, IgA, IgM, or C3 levels were found. Multiple comparisons revealed that there were no significant differences between patients with ALS and other neurodegenerative diseases in IgG, IgA, IgM, C3, or C4 levels. In addition, the IgG levels were lower in early-onset ALS patients than in late-onset ALS patients and HCs. The correlations between age at onset of ALS and IgG and IgA levels were significantly positive. Moreover, spinal-onset ALS patients had lower serum IgG levels than did HCs, but no difference was found between bulbar-onset ALS patients and HCs.

Conclusions: Peripheral immunity abnormalities existed in patients with ALS, and lower IgG levels were associated with early-onset ALS.

Background

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by the progressive degeneration of upper and lower motor neurons at the motor cortex, bulbar, and spinal levels, leading to muscle weakness, progressive paralysis, and respiratory failure and death within 3–5 years after disease onset [1]. Whilst the aetiology and pathology of ALS remains unclear, current evidence suggests that oxidative stress, glutamatergic toxicity, ribonucleic acid (RNA) processing dysfunction, abnormal protein aggregation, mitochondrial dysfunction, axonal transport dysfunction, neuroinflammation, and immunomodulation might play important roles in the pathogenesis of ALS [2, 3].

Recently, evidence indicating that there is a significant contribution of inflammatory and immune responses to the progression of ALS has emerged [4]. The activation of astrocytes and microglia and the infiltration of T cells and monocytes have been observed in the central nervous system (CNS) of patients with ALS and animal models [5–8]. Immunoglobulin deposits were observed in motor neurons from the

spinal cord of patients with ALS [9], and upregulated complements were found in the cerebrospinal fluid (CSF), spinal cord, and motor cortex of patients with ALS and transgenic mice [10–12]. In addition to the dysregulation of immune responses in the CNS, immune aberrations within the peripheral nervous system have been reported in patients with ALS. The levels of circulating chemokines and cytokines, such as IL-7, IL -6, and TNF-alpha and its soluble receptors, TNF-R1 and TNF-R2, were found to be increased in patients with ALS [13–15]. Increased levels of complement C3 and C5a, C reactive protein (CRP), and erythrocyte sedimentation rate (ESR) as well as T cell abnormalities and immunoglobulin alterations were found in the blood of patients with ALS [16–21]. However, some results regarding serum immunoglobulin and complement are inconsistent. Some studies have demonstrated significantly higher IgG levels, not IgM or IgA levels, in patients with ALS than in controls [22, 23]. Other studies have reported normal serum levels of IgG, IgA, and IgM [24, 25], or increased IgM levels and normal IgG level at the early stage of ALS, with a decrease in IgG levels and serum IgM level normalization with disease progression [26]. Additionally, although there is strong evidence of complement upregulation in ALS, normal C3 and C4 levels were also observed in patients with ALS [24, 27], and the specific genetic deletion of C3 and C4 in ALS mouse models did not show any beneficial effects on the disease progression [12, 28].

Neuroinflammation and immunomodulation have been hypothesized to play roles in the pathogenesis of ALS as well as other neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple system atrophy (MSA) [29–36]. Important differences in the immunological processes occurring in ALS, PD, and AD involving microglial function have been reported. Furthermore, although the contribution of adaptive immune cells to AD seems to be modest, T cells can influence microglial phenotypes and induce neuroprotection in PD, and especially in ALS models [33]. In addition, an increasing number of studies on peripheral immune component levels in patients with other neurodegenerative diseases, such as AD and PD, have been conducted [37, 38]; however, few studies have explored whether the contribution of peripheral immune components is similar among patients with ALS and other neurodegenerative diseases.

To evaluate these conflicting findings, resolve any discrepancies, and explore the relationships between ALS clinical characteristics and peripheral immune components as well as determine whether ALS shares similar peripheral immune abnormalities with other neurodegenerative diseases, the current study was designed: (1) to detect differences, if any, in the serum levels of IgG, IgM, IgA, and complement C3 and C4 among patients with ALS, patients with other neurodegenerative diseases, and healthy controls (HCs); (2) to systematically review the results of previous studies using the meta-analysis methodology; and (3) to compare the serum immune component levels between different ALS patient groups established by age at onset, site at onset, disease duration, or disease severity, and analyse the correlations of serum immune components with age at onset, disease duration, and the revised ALS functional rating scale (ALSFRS-R) score.

Subjects And Methods

Subjects

A total of 245 patients with ALS in the Department of Neurology in Xiangya Hospital at Central South University (CSU) in China from April 2013 to April 2019 were enrolled in the study. All patients were diagnosed with definite, probable, or probable-laboratory-supported ALS by at least two neurologists according to the revised El Escorial criteria 2015 [39]. Their demographic features and clinical data, including sex, age, site at onset, age at onset, disease duration, and the ALSFRS-R score, were collected. All data were collected when the blood tests were performed.

Two control groups were included in the study. A total of 82 sex- and age-matched healthy individuals without any neurological diseases were recruited from the Health Management Center in Xiangya Hospital at CSU as HCs. In addition, 65 sex- and age-matched patients with MSA and 60 matched patients with PD recruited from the same location as the ALS patients were enrolled as neurological disease controls. All patients with PD were diagnosed according to the Movement Disorder Society (MDS) clinical diagnostic criteria [40]. All patients with MSA were diagnosed according to the current consensus criteria established by Gilman and colleagues [41].

Subjects with systemic inflammation, monoclonal gammopathy, non-malignant endocrine abnormalities, neoplastic disorders, auto-antibodies, and infection, or those who had a history of using anti-inflammatory drugs, acetylsalicylic acid, steroids, and statins within the 2 months before enrolment were excluded from this study. This study was approved by the Ethics Committee of Xiangya Hospital at CSU in China, and all subjects included provided written informed consent prior to participation.

Biochemical methods

Ten milliliters of peripheral venous blood samples were collected from all subjects in accordance with the study protocols. Then, each blood sample that was collected was sent to the Clinical Laboratory at Xiangya Hospital, and the levels of immunoglobulin and complement were measured.

Statistical analysis

All analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 5.03 (® GraphPad Software, Inc.). Differences with $p < 0.05$ were considered statistically significant, and multiple comparisons were performed with Bonferroni correction. All continuous data, including those of age, disease duration, age at onset, the ALSFRS-R scores, and the levels of IgG, IgA, IgM, C3, and C4 are presented as means $\pm$ standard deviations. The Chi-square test was used to analyse the differences in the sex distributions among different groups. A one-way analysis of variance was used to analyse the differences in age, disease duration, age at onset, the ALSFRS-R scores, and the levels of IgG, IgA, IgM, C3, and C4 among more than two groups; then, Dunn's multiple comparison test was performed. The correlations between serum immune variables and ALS clinical characteristics, including age at onset, disease duration, and the ALSFRS-R score, were analysed using Spearman's correlation analysis.

Meta-analysis

The methods of search strategy, inclusion and exclusion criteria, outcome measures, and statistical analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)

statement [42]. The Embase, Pubmed, and the Cochrane Library primary databases were searched using the following search terms: “amyotrophic lateral sclerosis” OR “ALS” OR “motor neuron disease” OR “MND” combined with the terms “immunoglobulin” OR “IgG” OR “IgM” OR “IgA” OR “complement” OR “C3” OR “C4”. The language of the articles was restricted to English, and all searches were performed prior to April 6, 2020.

All the articles were carefully read and evaluated. Studies were included if they met the following criteria: (1) observational studies (including case-control studies or cohorts) that evaluated the serum levels of immunoglobulin or complement between patients with ALS and subjects without neurological diseases; (2) the ALS diagnostic criteria were clearly stated; and (3) met at least six points of the Newcastle-Ottawa Scale criteria [43], which was used to evaluate the methodological quality of case-control and cohort studies. Review articles, editorials, commentaries, case reports, animal experiments, hypothesis papers, letters that reported no new data, meta-analyses, and abstracts were excluded.

Two investigators independently selected studies and extracted data according to the inclusion criteria, and a third researcher was asked to resolve disputes. The following data were extracted: the first author, publication year, country, sample size, mean age of the study group, mean and standard deviations of the serum levels of immunoglobulin and complement, methods used to measure the levels of immunoglobulin or complement measurement.

In the meta-analysis, the primary summary measure was the standardized mean difference (SMD) in serum immunoglobulin and complement levels between patients with ALS and control subjects. The SMDs with 95% CIs were calculated using a random-effects model and presented in a forest plot for each immunity-related variable. The level of heterogeneity across studies was evaluated using the I^2 statistic [44] and Galbraith graph, and sensitivity analysis was performed by study-by-study exclusion. Publication bias was assessed using funnel plots, as well as Egger’s and Begg’s tests. A two-tailed p value of less than 0.05 was considered statistically significant. All data analyses were performed using STATA (version 13).

Results

Demographic and clinical features of subjects

The demographic and clinical features of the subjects are summarized in Table 1. There were no significant differences between patient groups and the HC group with regard to age and sex. For the ALS group, the average age at onset was 54.56 ± 9.71 years (range 25–73 years), the average disease duration for ALS was 16.20 ± 16.59 months with a mean ALSFRS-R score of 39.20 ± 5.64 , and 52 patients (21.2%) had with bulbar-onset symptoms.

Table 1
Characteristics of patients with ALS, MSA, and PD and HCs.

	ALS	MSA	PD	Healthy controls	<i>P</i> value
Number	245	65	60	82	
Age (years)	55.87 ± 9.71	58.02 ± 7.95	57.08 ± 10.49	54.98 ± 7.547	0.186
Sex, n (%)					0.213
Male	162(66.1%)	40	31	50(61.0%)	
Female	83(33.9%)	25	29	32(39.0%)	
Age at onset (years)	54.56 ± 9.71		-	-	
Disease duration (months)	16.20 ± 16.59		-	-	
ALSFRS-R score	39.20 ± 5.64		-	-	
Onset site, n (%)					
Spinal	193(78.8%)		-	-	
Bulbar	52(21.2%)		-	-	
Values are showed as mean (± standard deviation) or percentage.					

Serum levels of immunoglobulin and complement in patients with ALS, MSA, and PD, and HCs

There were no significant differences in the levels of IgA, IgM, or C4 among the patients with ALS, MSA, and PD and HC (Additional file 1). The levels of C3 were observed to be lower in the serum of the patients with MSA than in that of the HCs. No significant differences in serum C3 levels were found between the ALS patients and HCs, PD patients and HCs, ALS patients and MSA patients, or ALS patients and MSA patients. The patients with ALS had lower serum IgG levels than did the HCs. No significant differences in serum IgG levels existed between the MSA patients and HCs, PD patients and HCs, ALS patients and MSA patients, or ALS patients and MSA patients.

Meta-analysis comparing the serum levels of immunoglobulin and complement between patients with ALS and controls

After a systematic search and evaluation were performed, the results of eight published articles [23–26, 45–48] together with our results, representing data from 711 patients with ALS and 477 controls, met the inclusion criteria for a meta-analysis of IgG (Additional file 2). The pooled results suggested there were no

differences in serum IgG levels between patients with ALS and controls (Fig. 1). Five studies [24, 25, 45, 46, our study], including a total of 621 patients with ALS and 368 controls, were included in the meta-analysis of IgA (Additional file 2), and no significant differences in serum IgA levels between patients with ALS and controls were identified (Fig. 2). The pooled results from seven studies [23–26, 45, our study], including a total of 667 patients with ALS and 401 controls (Additional file 2), showed no significant differences in serum IgM levels between patients with ALS and controls (Fig. 3). The pooled results from four studies [24, 45, 47, our study], representing 554 patients with ALS and 327 controls (Additional file 2), suggested that patients with ALS had higher serum C4 levels than did controls (Fig. 4). The pooled results from three studies [24, 46, our study], including a total of 572 patients with ALS and 321 controls (Additional file 2), did not show any differences in serum C3 levels between patients with ALS and controls (Fig. 5). The process of screening studies in the literature, the characteristics of the studies included in the meta-analysis, and the heterogeneity analysis, sensitivity analysis and publication bias results are shown in Additional file 2 and Additional file 4.

The relationship between serum immunoglobulin and complement and clinical characteristics of ALS

We compared serum levels of immunoglobulin and complement between patients with early- and late-onset ALS, and the groups were established according to whether the age at onset of ALS was younger or older than 55 years of age. As shown in Fig. 6a, we observed differences in only serum levels of IgA, and no differences in IgG, IgM, C4, or C3 between patients with early-onset and late-onset ALS. We also compared the serum immunoglobulin and complement levels between early-onset or late-onset ALS patients and HCs. Interestingly, the serum levels of IgA in the early-onset ALS patients were lower than those in the HCs, while no differences were found between the late-onset ALS patients and HCs. Comparisons in IgG, IgM, C4, and C3 serum levels between early-onset or late-onset ALS patients and HCs showed similar results to the comparisons between the entire sample population of ALS patients and HCs.

In addition, the levels of immunoglobulin and complement were compared between ALS patients with short and long disease durations, who were grouped by whether their disease duration of ALS was longer than 12 months, or between mild and moderate-to-severe ALS patients, who were grouped according to whether their the ALSFRS-R score was > 36. As shown in Fig. 6b and 6d, no significant differences in the levels of immunoglobulin and complement were found between patients with short and long disease durations of ALS or between mild and moderate-to-severe ALS patients. Additionally, the comparisons of the serum levels of immunoglobulin and complement between the ALS subgroups established by disease duration or disease severity and the HCs showed similar results to the comparisons between the entire sample population of ALS patients and HCs.

ALS patients were also divided into two groups according to site at onset (bulbar onset or spinal onset). As shown in Fig. 6c, no differences in the serum levels of immunoglobulin and complement were found between these two subgroups. Notably, although the spinal-onset ALS patients had lower levels of IgG

than did the HCs, no differences in the serum levels of IgG between bulbar-onset ALS patients and HCs were identified. In addition, the comparisons of the serum levels of IgA, IgM, C4, and C3 between the ALS subgroups established by the site at onset and HCs showed similar results with the comparisons between the entire sample population of ALS patients and HCs.

Sex, age, age at onset, disease duration, and the ALSFRS-R score have been adjusted when the ALS patients subgroups were established according to age at onset, site at onset, disease duration, or disease severity were controlling. The concentrations of immunoglobulin and complement, sexes, ages, ages at onset, disease durations, and ALSFRS-R scores for different subgroups are summarized in Additional file 3.

Table 2
Correlation analysis between the serum levels of complement and immunoglobulin and clinical characteristics of ALS.

	Age at onset		Disease duration		ALSFRS-R score	
	rs	P value	rs	P value	rs	P value
C3	-0.042	0.508	0.014	0.825	-0.008	0.901
C4	0.088	0.170	0.025	0.703	-0.059	0.354
IgA	0.158	0.013	-0.035	0.591	-0.003	0.963
IgG	0.130	0.041	0.026	0.684	0.001	0.982
IgM	-0.091	0.154	-0.083	0.196	0.031	0.632
rs denotes correlation coefficients.						

As shown in Table 2, age at onset was significantly positively correlated with the serum levels of IgA and IgG. No significant correlations were observed between age at onset and IgM, C3, or C4. In addition, no significant correlations were found between the ALSFRS-R score or disease duration and the serum levels of immunoglobulin and complement.

Discussion

In the present study, a meta-analysis including our data indicated that the serum levels of IgG, IgA, IgM, and C3 in patients with ALS did not differ significantly from those in HCs, while the serum levels of C4 were higher in ALS patients than in HCs. Although differences in the serum levels of immunoglobulin and complement between patients with ALS and HCs have been reported in several studies, some of the results are conflicting. For example, several studies have reported that the levels of IgG are higher in patients with ALS than in controls [23, 48]. No differences in IgG levels between patients with ALS and HCs were also reported [24, 25]. In addition, lower levels of IgG in patients with severe and moderate ALS [24, 26] as well as mild ALS (our study) than in controls were observed. The discrepancy may be related

to the heterogeneity of the patient cohorts and the limited number of patients. One way to address this problem is to combine all available published data in a systematic meta-analysis. To our knowledge, our current study is the first meta-analysis conducted to draw systematic conclusions from these inconsistent results. The pooled results in the present study supported the hypothesis that peripheral complement activation exists in the ALS patients. In addition, the levels of C4d and C4 in the CFS and the glial cells in the vicinity of motor neurons in the spinal cord and motor cortex were observed to be higher in ALS patients than in HCs [36, 49], providing evidence of the complement C4 activation in the CNS of ALS patients. Therefore, the classic complement pathway may be involved in the pathophysiology of ALS. However, where these complement factors originate and what initiates their activation remain unknown.

Although patients with MSA tend to have lower serum C3 levels than do HCs and lower serum C3 levels were not observed in patients with ALS and PD, no differences in the serum levels of immunoglobulin and complement were observed between ALS and MSA patients or between ALS and PD patients. In addition, certain shared pathogenic features involving macrophage activation in the blood of patients with ALS and other neurodegenerative diseases have also been reported [26]. Therefore, ALS seems to share similar features with other neurodegenerative diseases with regard to peripheral immunity activation.

In the present study, no differences in the serum levels of immunoglobulin and complement were observed between mild and moderate-to-severe ALS patients, and the results of the comparisons of the serum levels of immunoglobulin and complement between mild or moderate-to-severe ALS patients and HCs were the same as those of the comparisons between all the ALS patients in the study population and HCs. In addition, the correlation analysis did not show any significant correlations between the ALSFRS-R score and the serum levels of immunoglobulin and complement. Our data indicated that the levels of serum immunoglobulin and complement did not vary with disease severity, which was consistent with the results in a previous study that showed no significant correlations between immunoglobulin levels and the ALSFRS-R score [25]. However, Zhang et al. [26] demonstrated that no significant differences in IgG levels existed between patients with mild and moderate ALS and normal controls; however, lower levels of IgG were found in patients with severe ALS (ALSFRS-R ≤ 24) compared to normal controls. In addition, the authors found that the levels of serum IgM in patients with mild-to-moderate ALS were significantly higher than those in normal controls, but there were no differences between patients with severe ALS and normal controls. Similarly, Chen et al. [24] found that the levels of IgG were lower in ALS patients with moderate (ALSFRS-R range 25–36) and severe (ALSFRS-R range 0–24) impairment than in those with mild (ALSFRS-R range 37–48) impairment. They also revealed a significantly positive correlation between the ALSFRS-R scores and levels of IgG. One reason for the discrepancy may be that age, sex, disease duration, and age at onset were strictly controlled when ALS patients were divided into mild and moderate-to-severe groups in the present study. Another reason may be that there was heterogeneity in the patient cohorts.

We observed that serum levels of IgG, IgA, IgM, C4, and C3 did not vary or correlate with disease duration. Similarly, Rentzons et al. [25] reported no significant correlations between serum immunoglobulin levels

and disease duration in patients with ALS. However, Chen et al. [24] identified a positive correlation between IgG serum levels and disease duration. This inconsistency may be related to the heterogeneity of the patient cohorts.

We did not observe any differences in the serum levels of immunoglobulin or complement between spinal-onset and bulbar-onset ALS patients, which agrees with the observations from a previous study demonstrating that the levels of IgG, IgA, and IgM in serum do not differ significantly in ALS patients with or without bulbar-onset [25]. Notably, we found that the spinal-onset ALS patients had lower serum IgG levels than did the HCs, but no differences were observed between the bulbar-onset ALS patients and HCs. This results indicates that bulbar-onset ALS may have a potentially unique pathogenesis pathway compared with that of spinal-onset ALS. In fact, differences in pathology between bulbar-onset and spinal-onset ALS patients have been reported. For example, some bulbar-onset ALS cases presented with atypical pathology such as neurofibrillary tangles and basophilic inclusion, which were not found in the spinal-onset ALS cases [50].

Additionally, we found that the serum levels of IgA were lower in patients with early-onset ALS than in patients with late-onset ALS and HCs. An additional correlation analysis revealed that the serum levels of IgA and IgG were positively correlated with age at onset. Our data indicated that lower levels of IgA and IgG might contribute to the early onset of ALS. A recent study suggested that Chinese patients with sporadic ALS have a relatively long survival time, probably due to their young age and the small number of bulbar-onset cases [51]. Therefore, we hypothesized that ALS patients with lower serum IgG levels may have a longer survival time due to their younger age at onset and the form of the disease more commonly being spinal onset.

Conclusions

This study validated the existence of peripheral immune abnormalities in ALS patients and explored the association between peripheral immunity and the clinical characteristics of ALS. We also demonstrated that ALS may share similar features with PD and MSA with regard to peripheral immunity activation. In addition, our data suggest that lower IgG levels might be related to the early onset and spinal onset of ALS and might contribute to the longer survival time of Chinese ALS patients. The results of our studies provide additional evidence for researchers on the pathogenesis of ALS and shed light on potential therapeutic interventions aimed at modulating immunity.

Abbreviations

AD: Alzheimer's disease

ALS: amyotrophic lateral sclerosis

ALSFRS-R: revised ALS functional rating scale

CNS: central nervous system

CRP: C reactive protein

CSF: cerebrospinal fluid

CSU: Central South University

ESR: erythrocyte sedimentation rate

HCS: healthy controls

MDS: Movement Disorder Society

MSA: multiple system atrophy

PD: Parkinson's disease

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

RNA: ribonucleic acid

SMD: standardized mean difference.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Xiangya Hospital at CSU in China, and all subjects included provided written informed consent prior to participation.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MLW contributed to data analysis and wrote the paper. ZL, JD, YCY, BJ, and XWZ contributed to the collection of sample population and blood samples. XH, LS, JFG, HJ, KX, and BST supervised the research. JLW designed the experiment, revised the manuscript, supervised the research. All authors read and approved the final manuscript.

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Figures

Figure 1

Meta-analysis comparing the serum levels of IgG between patients with ALS and HCs. Pooled results suggesting no difference in the serum levels of IgG between patients with ALS and HCs

Figure 2

Meta-analysis comparing the serum levels of IgA between patients with ALS and HCs. Pooled results suggesting no difference in the serum levels of IgA between patients with ALS and HCs.

Figure 3

3 Meta-analysis comparing the serum levels of IgM between patients with ALS and HCs. Pooled results suggesting no difference in the serum levels of IgM between patients with ALS and HCs.

Figure 4

Meta-analysis comparing the serum levels of C4 between patients with ALS and HCs. Pooled results indicating higher serum levels of C4 in patients with ALS than in HCs.

Figure 5

Meta-analysis comparing the serum levels of C3 between patients with ALS and HCs. Pooled results suggesting no differences in the serum levels of C3 between patients with ALS and HCs.

Figure 6

Comparisons of the serum immunoglobulin and complement levels between different ALS patient groups and HCs. Multiple comparisons were performed with Bonferroni correction, and $p < 0.017$ was considered to be statistically significant in this analysis. Ns denotes no significance, * denotes $p < 0.017$, and ** denotes $p < 0.003$. (a) ALS patients grouped by age at onset: an age at onset of < 55 years old indicated early onset, and an age at onset of ≥ 55 years old indicated late onset. (b) ALS patients grouped by disease duration: a disease duration of ≥ 12 months indicated a long duration, and a disease duration of < 12 months indicated a short duration. (c) ALS patients grouped by site at onset: symptoms at onset involving only the spinal cord indicated spinal onset, and symptoms at onset involving the bulbar region indicated bulbar onset. (d) ALS patients grouped by disease severity: an ALSFRS-R score of ≥ 37 indicated mild ALS, and an ALSFRS-R score of < 37 indicated moderate-to-severe ALS.

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